CHAPTER 2

Penicillin-binding protein 4 of *Escherichia coli* shows a novel type of primary structure among penicillin-interacting proteins

H. Mottl, P. Terpstra & W. Keck

FEMS Microbiology Letters **78** (1991) 213-220.

SUMMARY

The nucleotide sequence of a 1884 bp DNA fragment of *E. coli*, carrying the gene *dac*B, was determined. The DNA codes for penicillin-binding protein 4 (PBP4), an enzyme of 477 amino acids, being involved as a DD-carboxypeptidase - endopeptidase in murein metabolism. The enzyme is translated with a cleavable signal peptide of 20 amino acids, which was verified by sequencing the amino-terminus of the isolated protein. The characteristic active-site fingerprints SXXK, SXN and KTG of class A ß-lactamases and penicillin-binding proteins were located in the sequence. On the basis of amino acid alignments we propose, that PBP4 and class A ß-lactamases share a common evolutionary origin but PBP4 has acquired an additional domain of 188 amino acids in the region between the SXXK and SXN elements.

INTRODUCTION

It has recently been proposed on the basis of crystallographic, enzymological and amino acid alignment data, that PBPs and ß-lactamases are evolutionarily related to each other and form a superfamily of penicillin-interacting proteins [1]. The ß-lactamases are divided into four classes, according to their sequence relationships. Class A, C and D act enzymatically by an active serine. Class B ß-lactamases are Zn^{2+}-enyzmes. Class A and C ß-lactamases show weak, but significant sequence similarity with the PBPs and DD-carboxypeptidases. This relationship is most pronounced around some active-site fingerprints. We became interested in how PBP4 could be grouped into this superfamily and how its properties are reflected in its primary structure. In this paper we present the sequence of the DNA fragment that codes for PBP4. The deduced amino acid sequence of PBP4 is discussed with respect to the apparent solubility of the protein. Fingerprints specific for penicillin-interacting proteins were identified in the amino acid sequence of PBP4. Alignments with ß-lactamases of class A were performed and a proposal for the spatial organization of the protein into two domains is given.

MATERIALS & METHODS

Plasmid pBK18-1 is a recombinant pUC18 derivative, carrying a 1884 bp *Sma*I/*Eco*RI *dac*B fragment [2]. All enzymes were from Boehringer Mannheim, radiolabelled chemicals were from Amersham. All other chemicals were from Merck, if not otherwise stated. Phages M13mp10, M13mp11 and their host JM101 are described in [3]. All recombinant DNA techniques were performed as described [4]. The DNA fragment was sequenced using the *Bal*31-exonuclease approach exactly as described by Davis [4]. Briefly: the plasmid pBK18-1 was linearized with *Eco*RI or *Hind*III whithin the polylinker, and digested with exonuclease. The shortened *dac*B fragments were excised with *Hind*III or *Eco*RI and cloned into the *Hind*III-*Sma*I or *Eco*RI-*Sma*I sites of M13mp10 and M13mp11. The resulting recombinant M13 clones were ordered by size and sequenced as described [4]. Both strands of the DNA fragment were sequenced at least twice.

The amino-terminal sequencing was performed with 1 nmol PBP4, which was isolated as described [2]. The protein was electrotransferred from a 10% SDS-polyacrylamide gel onto a Poly-vinylidenedifluoride membrane [5] and sequenced directly in an Applied Biosystems 477A pulse liquid amino acid sequencer

connected to a C120A PTH-derivative analyzer (Eurosequence, Groningen, The Netherlands). Polyacrylamide gel-electrophoresis and electroblotting were done as described earlier [2].
DNA and Protein sequences were analysed with the PC-Gene collection of programs from Genofit (Geneva, Switzerland). The protein Identification Resource Database was screened with the program FASTP [6]. Alignments of proteins were done, using the programs ARGOS [7] and GOAD/ KANEHISHA [8] for pairwise alignments and CLUSTAL [9] for multiple alignments. The programs were run on a VAX 750.

RESULTS

Sequencing

The sequenced fragment codes for an open reading frame of 477 amino acids which begins at bp 140 and stops at bp 1570 (Fig. 1). Using a promoter identification program written by P. Terpstra which is based on a search matrix described in [10], we propose a promoter which was classified as medium strong upstream from the predicted ORF, with the -35 region starting at bp 101 and the -10 region at bp 77 (Fig.1). We were not able to detect a ribosome binding site using the programm described in [11]. Immediately downstream of the ORF lies an inverted repeat, with a stem of 7 bp and a loop of 5 bp (the free energy of stabilization is -21.4 kcal) which could function as a ρ-independent transcription terminator.

Characterization of the amino acid sequence

(i) Membrane translocation signal

Penicillin-binding proteins are located in the periplasmic space on the outer side of the cytoplasmic membrane and so we expected to find an amino-terminal signal peptide which would mediate the translocation of the protein across the cytoplasmic membrane. Using von Heijne's algorithm [12] the amino-terminal 20 amino acids satisfy the criteria including a potential cleavage site between Ala +1 and Ala -1. The prediction was confirmed by sequencing the first eleven amino acids of the purified mature enzyme. PBP4 is synthesized as a preprotein of 477 amino acids and processed to a protein of 457 amino acids after transport across the cytoplasmic membrane (Fig.1).

(ii) Membrane anchor

All PBPs of *E. coli* are described as membrane bound enzymes, that are anchored by a transmembrane helix [13], an amphiphilic helix [14] or a lipid anchor [15] in the cytoplasmic membrane. We reported, that after overproduction of the cloned PBP4 most of the protein can be detected in the 100,000 x g supernatant of a cell free extract [2]. Therefore we carefully screened the sequence for transmembrane helices, lipid attachment sites and amphiphilic membrane anchors. No transmembrane helices could be detected, using the methods described in [16,17], nor could we locate a carboxy-terminal surface seeking amphiphilic helix by the method of Eisenberg [18]. Finally no attachment site for a lipid anchor [19] could be detected.

(iii) Characteristic active-site fingerprints

Two characteristic amino acid fingerprints are found in all PBPs and ß-lactamases of class A,C and D. The first fingerprint is formed by the active-site serine with the invariable sequence SXXK. This element is located between amino acid 40 and 60 at the amino-terminus of the enzyme or penicillin-binding domain. The second fingerprint reads KTG.(KSG, HTG are also found. For the sake of simplicity this element will here only be referred to as KTG). It is located around 60 amino acids before the carboxy- terminus of the enzyme [1]. A third fingerprint, which will here be referred to as SXN lies around 80 amino acids carboxy-terminal to the active-site serine and is found in all penicillin-interacting enzymes (Table 1.) Inspection of the known tertiary structures of penicillin-interacting enzymes reveals that the above mentioned boxes form part of the active-site cleft [20,21]. The overall primary structure similarity in penicillin-interacting enzymes is rather low,

```
          CCCGGGTACAAGTCCCAGGTCAGCTACAATTCACATTTTGATAGTCATTTTACCCTGAAGTTCCCGA   67
AGGTCATCGTTTACTTTATAGGGCGTTGCGCCGTAGTATGACGGCTCGATTCCAGGTTGTTAGCGCGAGATT  139

ATGCGATTTTCCAGATTTATCATCGGATTGACCAGCTGTATAGCGTTCAGTGTTCAGGCCGCAAATGTTGAT  211
-20 M  R  F  S  R  F  I  I  G  L  T  S  C  I  A  F  S  V  Q  A▲A  N  V  D

GAGTACATTACTCAACTCCCCGCTGGTGCCAACCTTGCCCTGATGGTGCAAAAAGTCGGCGCGTCGGCCCCC  283
  5 E  Y  I  T  Q  L  P  A  G  A  N  L  A  L  M  V  Q  K  V  G  A  S  A  P

GCTATTGATTACCACAGTCAGCAGATGGCGCTGCCTGCCAGTACCCAGAAAGTGATTACTGCGCTGGCGGCG  355
 29 A  I  D  Y  H  S  Q  Q  M  A  L  P  A  S  T  Q  K  V  I  T  A  L  A  A

TTGATTCAACTCGGCCCCGATTTTCGTTTTACCACGACGCTTGAAACCAAAGGCAATGTGGAAAACGGCGTA  427
 53 L  I  Q  L  G  P  D  F  R  F  T  T  T  L  E  T  K  G  N  V  E  N  G  V

CTTAAGGGTGACTTAGTGGCGCGATTTGGTGCCGATCCGACGTTAAAACGTCAGGATATTCGCAATATGGTC  499
 77 L  K  G  D  L  V  A  R  F  G  A  D  P  T  L  K  R  Q  D  I  R  N  M  V

GCGACTTTGAAAAAATCTGGCGTCAACCAAATCGATGGCAATGTGTTGATAGATACCTCCATTTTCGCCAGC  571
101 A  T  L  K  K  S  G  V  N  Q  I  D  G  N  V  L  I  D  T  S  I  F  A  S

CACGATAAAGCCCCCGGCTGGCCATGGAATGACATGACACAATGCTTTAGCGCTCCGCCTGCCGCCGCCATA  643
125 H  D  K  A  P  G  W  P  W  N  D  M  T  Q  C  F  S  A  P  P  A  A  A  I

GTTGACCGCAACTGTTTCTCCGTCTCGCTCTACAGTGCCCCAAAGCCTGGTGATATGGCTTTTATACGCGTG  715
149 V  D  R  N  C  F  S  V  S  L  Y  S  A  P  K  P  G  D  M  A  F  I  R  V

GCATCTTATTACCCCGTTACGATGTTCAGCCAGGTACGCACCCTCCCCCGTGGTTCTGCCGAAGCGCAATAC  787
173 A  S  Y  Y  P  V  T  M  F  S  Q  V  R  T  L  P  R  G  S  A  E  A  Q  Y

TGCGAACTGGATGTGGTGCCAGGCGACCTGAACCGCTTTACGCTGACGGGATGCCTGCCACAACGTTCTGAG  859
197 C  E  L  D  V  V  P  G  D  L  N  R  F  T  L  T  G  C  L  P  Q  R  S  E

CCGCTCCCGTTGGCTTTTGCCGTGCAGGATGGAGCCAGCTATGCCGGTGCAATTCTGAAAGATGAGTTAAAA  931
221 P  L  P  L  A  F  A  V  Q  D  G  A  S  Y  A  G  A  I  L  K  D  E  L  K

CAGGCGGGTATCACCTGGAGCGGAACACTGCTGCGCCAGACTCAGGTTAACGAACCTGGAACGGTAGTTGCC 1003
245 Q  A  G  I  T  W  S  G  T  L  L  R  Q  T  Q  V  N  E  P  G  T  V  V  A

AGTAAACAGTCGGCCCCGCTGCACGATCTGCTTAAGATTATGCTGAAAAAGTCGGACAACATGATCGCCGAT 1075
269 S  K  Q  S  A  P  L  H  D  L  L  K  I  M  L  K  K  S  D  N  M  I  A  D

ACGGTTTTCCGCATGATAGGCCATGCGCGCTTCAATGTGCCTGGAACATGGCGGGCCGGGTCGGACGCCGTG 1147
293 T  V  F  R  M  I  G  H  A  R  F  N  V  P  G  T  W  R  A  G  S  D  A  V

CGTCAGATCCTGCGCCAGCAAGCCGGTGTCGATATTGGAAACACCATTATTGCCGATGGTTCAGGGCTTTCG 1219
317 R  Q  I  L  R  Q  Q  A  G  V  D  I  G  N  T  I  I  A  D  G  S  G  L  S

CGGCATAACCTGATTGCCCCCGCCACCATGATGCAGGTGCTGCAATACATTGCCCAACACGACAATGAACTT 1291
341 R  H  N  L  I  A  P  A  T  M  M  Q  V  L  Q  Y  I  A  Q  H  D  N  E  L

AACTTTATCTCCATGCTGCCGCTGGCGGGCTATGACGGCTCTTTGCAGTACCGTGCAGGTCTGCATCAGGCG 1363
365 N  F  I  S  M  L  P  L  A  G  Y  D  G  S  L  Q  Y  R  A  G  L  H  Q  A

GGCGTGGATGGAAAAGTCTCAGCGAAAACCGGTTCGTTGCAGGGGGTATATAACCTGGCGGGATTCATTACC 1435
389 G  V  D  G  K  V  S  A  K  T  G  S  L  Q  G  V  Y  N  L  A  G  F  I  T

ACAGCGAGCGGGCAACGAATGGCGTTTGTGCAATATCTTTCTGGCTATGCAGTAGAACCTGCGGATCAGCGT 1507
413 T  A  S  G  Q  R  M  A  F  V  Q  Y  L  S  G  Y  A  V  E  P  A  D  Q  R

AATCGCCGTATTCCGTTAGTGCGTTTTGAAAGCCGTTTGTATAAAGATATTTATCAGAACAATTAGTCAAAA 1579
437 N  R  R  I  P  L  V  R  F  E  S  R  L  Y  K  D  I  Y  Q  N  N  ♦♦♦

AGAAACCCCGGCACATGGCCGGGGCTTCAGATTATTGACAAAGTGCGCTTTGTTTATGCCGGATGCGGCGTAAACGC 1656
CTTATCCGGCCTACAAAATCGTGCAAATTCAACATATTGCAATTCTCTTGTAGGCCTGATAAGCGTAGCGCATCAGG 1733
GTGATTTGGCGTTTATCATCAGTGATTAACGCTTGTAAATGAACTCAACGCCTTCTTCGTCGTCTTCGTCCCAGTCG 1810
TCATCCCAGTCTTCATCATCCTCTTCAGCAATCTCTTCAAGCTGCTGGCGATGATAATCATCCCACATGAATTC
```

FIG. 1 The nucleotide sequence and the deduced amino acid sequence of the 1884 bp *Sma*I-*Eco*RI fragment containing the *dac*B gene of *E. coli*. The open reading frame (bp 140-1570) codes for a protein of 477 amino acids with a calculated M_r of 51,798 Daltons. The first 20 amino acids form a signal peptide which is cleaved off. This is indicated by an arrow at position -1/+1. Amino acid sequencing verified the underlined amino terminus of the mature protein. The calculated M_r of 49,568 is in good agreement with the M_r of PBP4 derived from SDS-polyacrylamide gel electrophoresis, which was found to be 49,000. The proposed promoter at bp 77: -35 region and bp 101: -10 region is underlined as well as the inverted repeat (bp 1585-1603). The fingerprints SXXK, SXN and KTG at amino acids 42-45, 286-288 and 397-399 are printed in bold letters. These sequence data were submitted to the EMBL/Gen Bank/DDBJ Nucleotide Sequence Data Libraries under the accession number X60038 E. COLI, DACB, DNA.

Table 1. Distance of active-site fingerprints in PBPs and β-lactamases.

Enzymes	Distances of conserved residues		
	SXXK→KTG	SXXK→SXN	SXN→KTG
β-Lact. Class A	161-169	57-65	103
β-Lact. Class C	250	(85)	(164)
R61	235	(96)	(138)
PBP1A	250	58	191
PBP1B	187	61	125
PBP2	214	96	117
PBP3	186	51	134
PBP5/6	168	65	102
PBP4	**354**	**243**	**110**

Amino acid distances were calculated between the serine residue of SXXK, the serine residue of SXN and the lysine residue of KTG. Numbers in parentheses refer to distances between YAN or YSN and SXXK or KTG respectively. The sequences of class A ß-lactamases were taken from Fig.3. The sequences of the class C ß-lactamases from *E. coli* and *C. freundii*, the sequence of the *Streptomyces* R61 DD-carboxypeptidase and the sequences of *E. coli* PBPs 1A to 3 were taken from [1]. The sequences of PBP5 and PBP6 were from [27]. The exact location of the fingerprints are : **R61**: S62VTK, Y159SN, H298TG. **PBP1A**: S465NIK, S524KN, K716TG. **PBP1B**: S510LAK, S572MN, K698TG. **PBP2**: S329TKK, S426GN, K544SG. **PBP3**: S307TVK, S359SN, K494TG. **PBP5**: S44LTK, S110GN, K213TG. **PBP6**: S39LTK, S105GN, K208TG. **PBP4**: S42TQK, S286DN, K397TG. *E. coli* **BlaC**: S80VSK, Y166AN, K331TG.

but the tertiary structures of different enzymes resemble one another and this holds especially true for the scaffolding of their active site cavity [22,23]. The active serine is always located at the amino-terminus of an α-helix, the KTG element forms part of a β-sheet and the SXN box is part of a loop which protudes into the catalytic center. The serine of the SXXK fingerprint performs the nucleophilic attack on the ß-lactam or DD-peptide bond. The reason for the strict conservation of the lysine in the SXXK fingerprint has so far not been clearly established. From cocrystallyzation experiments of the *Streptomyces* R61 DD-carboxypeptidase and also the *Citrobacter freundii* Class C β-lactamase with ß-lactams it was claimed that this lysine forms together with the lysine of the KTG element a hydrogen bonding network, which serves to coordinate the C-3 carboxyl-group of ß-lactams and probably also the carboxyl-group in DD-peptides [21,24]. The main-chain atoms of the KTG fingerprint stabilize the bound ß-lactam or dipeptide through hydrogen-bonds. The function of the SXN element is not yet clear. Based on crystallographic data it has been proposed that in ß-lactamases of class C the sequence YAN is functionally and sterically identical with the SXN sequence, the crucial residues being the serine or tyrosine respectively, both hydroxy amino acids and that the tyrosine acts as a general base in the deacylation of the bound ß-lactam [21].

Visual inspection of the amino acid sequence of PBP4 identifies all three fingerprints (S42TQK, S286DN, K397TG). SXXK occurs three times in the sequence (S42, S124, S160). By comparison with other active-site serines and due to the criterion, that the active-site serine is located not far from the amino terminus Ser42 was proposed as the active-site serine. This prediction has in the meantime been confirmed by mutational analysis (H.Mottl, unpublished results). The

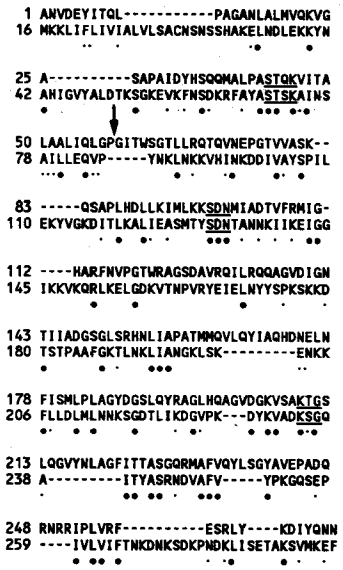


FIG. 2. Amino acid alignment of PBP4* with Staphylococcus aureus PC1 ß-lactamase. The upper sequence is PBP4*. A deletion of 188 amino acids (D59-A246) was introduced into the PBP4 sequence. The location of the deletion is marked with an arrow. The alignment was performed with the program ARGOS [7]. Large dots mark identical, small dots mark related amino acids. The three active-site fingerprints SXXK, SXN and KTG are underlined. The ß-lactamase is numbered according to Ambler [28]. The overall alignment score was 3,0. The scores around the active-site fingerprints were for SXXK: 3,8 for SXN: 4,7 and for KTG 3,8.

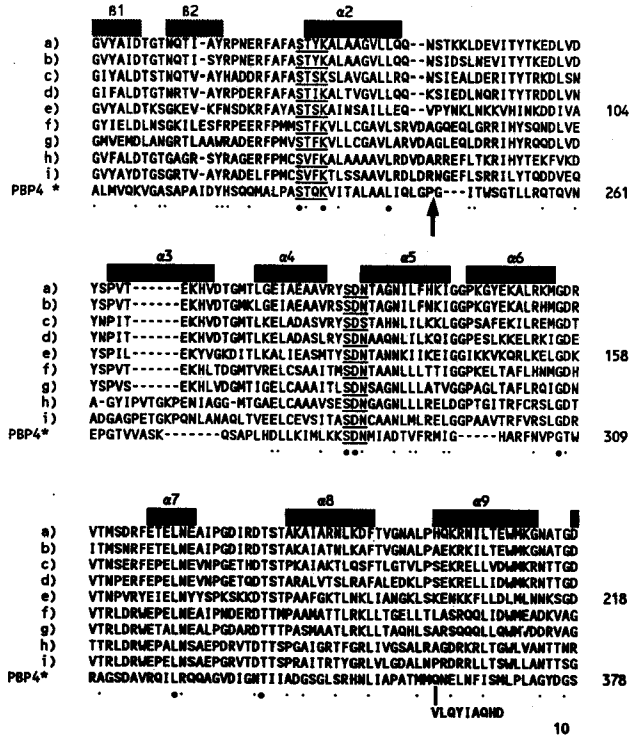
KTG fingerprint was located at amino acid 397 at the correct distance from the carboxy-terminus. This spacing of the active-site serine and the KTG fingerprint is unique among the PBPs. This distance is at least 135 amino acids in the case of a class D ß-lactamase and the maximum spacing observed is 251 amino acids in PBP1A of *E. coli*. In PBP4 the distance is 354 amino acids. The SXN element can be located at amino acid 286 and comparison of the distances between SXXK, SXN and KTG (Table 1) shows, that the spacing of SXN and KTG lies within the limits that were set by other penicillin-interacting enzymes. The unusual spacing in PBP4 has thus to be assigned to the region between the active-site serine and the SXN fingerprint.

(iv) Amino acid alignments

Considering the spacing of active-site residues in PBP4 we tried to learn more about the evolutionary relationship of PBP4 to other penicillin-interacting proteins.

When we used the complete amino acid sequence of PBP4 in comparisons with penicillin-interacting enzymes we were not able to align more than one fingerprint, no matter which PBP or ß-lactamase was chosen. We then introduced deletions between the SXXK and SXN fingerprints for the purpose of comparison. With this strategy we obtained an alignment in which all three fingerprints were correctly matched with the class A ß-lactamase of *Staphylococcus aureus* (Fig. 2) only when a 188 amino acid long fragment of the sequence was omitted in the PBP4 sequence between the SXXK and SXN fingerprint (D59 - A246). In this PBP4* 316 positions, including the introduced gaps were aligned, 55 positions were identical, 39 were homologous according to the Dayhoff criteria and 81 positions were occupied by gaps.

We then tried to reduce the noise in the alignment and to work out the essential homologies by simultaneously comparing the PBP4* sequence with a set of nine different class A ß-lactamases (Fig.3). The multiple alignment was carried out with the programm CLUSTAL [9]. Again we found that the active-site fingerprints were aligned. But the homology in the rest of the sequence was greatly reduced. In 260 aligned positions 10 are identical and 53 are homologous. When considering the distribution of these residues one finds seven of the identical and 22 of the homologous residues within ± 10 amino acids reach of the fingerprints. Outside the active-site fingerprints three conserved amino acids were detected: G307, L320 and T331.



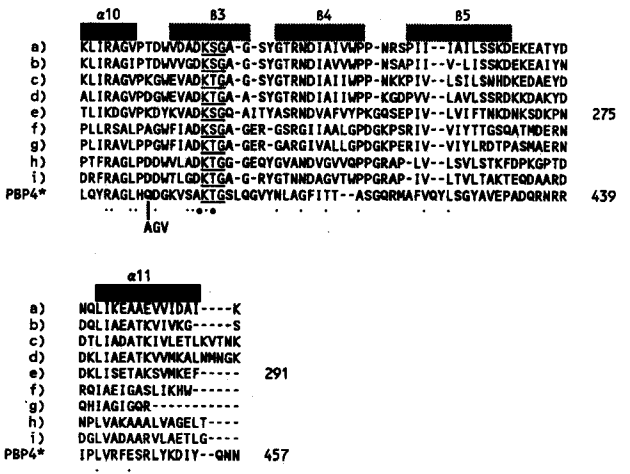


FIG. 3. Multiple alignment of PBP4* with ß-lactamases of class A. Key to the reference numbers a) to i): a) *Bacillus cereus* ß-lactamase PenPC, [29], b) *Bacillus cereus* 569/H ß-lactamase BlaY [30], c) *Bacillus cereus* 569/H ß-lactamase III BlaZ [31], d) *Bacillus licheniformis* ß-lactamase BlaP [32], e) *Staphylococcus aureus* PC1 ß-lactamase BlaZ [33], f) *Escherichia coli* ß-lactamase BlaC [34], g) *Klebsiella pneumoniae* LEN-1 ß-lactamase [35], h) *Streptomyces aureofaciens* ß-lactamase Bla (Reynes *et al.* submitted 1988 to EMBL data library under accession number P10509), i) *Streptomyces albus* G ß-lactamase BlaC [36]. For the purpose of comparison 188 amino acids (D59-A246) from the sequence of PBP4 were deleted, resulting in PBP4*. The point of deletion is marked by an arrow in the figure. The three fingerprints SXXK, SXN and KTG are underlined. PBP4* and the *S. aureus* ß-lactamase are numbered as in Figure 2. The sequences were truncated at the amino- and carboxy-termini since homology in these regions is not pronounced. Identical amino acids in all sequences are marked with large dots. Related amino acids in all sequences are marked with small dots. Above the sequences the secondary structure elements of the crystallized ß-lactamase from *Staphylococcus aureus* PC1 are indicated with solid and hatched boxes. Their nomenclature is derived from [26]. The alignment was performed with the program CLUSTAL [9]. Uniform and variable gap penalties were set to 20. Two small deletions were introduced into the sequence of PBP4*, the first one VLQYIAQHD at amino acid 352, the second AGV at amino acid 387.

DISCUSSION

The open reading frame of the *dac*B gene was identified on a 1884 bp chromosomal DNA *Sma*I-*Eco*RI fragment of *E. coli* that codes for penicillin-binding protein 4, a protein of 477 amino acids. Amino-terminal sequencing of the isolated protein showed, that the mature PBP4 is a protein of 457 amino acids with a cleaved signal-peptide of 20 amino acids and a resulting M_r of 49,568. This is in good agreement with the reported M_r of 49,000, as determined by SDS/PAGE. Although in the wild type situation the enzyme can be detected mainly in the membrane fraction, we found no motifs in the sequence which could function as a membrane anchor. In the preceding paper we showed, that after overproduction of PBP4 90% was found in the soluble 100,000 x g supernatant of a cell free extract [2]. PBP4 is thus, as we speculated, actually a soluble protein. That it can be detected in and isolated from the membrane fraction of an *E. coli* cell-free extract might be explained by a general affinity for membranes or proteins associated with the membrane or murein, yet clear evidence is missing. The absence of a consensus ribosome binding site is not completely unexpected, since the number of PBP4 proteins in the cell was estimated to be 110 copies [25]. The absence of a clearly recognizable ribosome binding site may explain the low expression rate. Analysis of the sequence of PBP4 showed, that PBP4 possesses the three fingerprints SXXK, SXN and KTG which are characteristic of PBPs. SXXK and KTG are conserved in all penicillin-interacting enzymes (with the exception of ß-lactamases of class B which are as metalloenzymes mechanistically distinct) whereas more variation is found in the SXN region. Class C ß-lactamases and the *Streptomyces* R61 DD-carboxypeptidase possess a homologous sequence YAN, where the tyrosine probably serves the same purpose as the homologous serine, as has been proposed [21]. The spacing of the active-site serine and the SXN fingerprint is extraordinarily long in PBP4, whereas the spacing between the SXN and the KTG is in the same range as found in other PBPs (Table 1). Using the complete sequence of PBP4 in computer assisted homology searches, no homology with other penicillin-interacting enzymes could be detected. However, when a consecutive stretch of 188 amino acids (D59-A246) was cut out between the SXXK and SXN region for the purpose of computer assisted alignments, we could produce correct alignments of the three fingerprints only with ß-lactamases of class A. This suggests that PBP4 and ß-lactamases of class A have a common ancestor and that PBP4 has acquired an additional domain of 188 amino acids (Fig.4). A screening of the Protein Identification Resource database for sequences which match with the 188 amino acid segment detected no highly significant relationships.

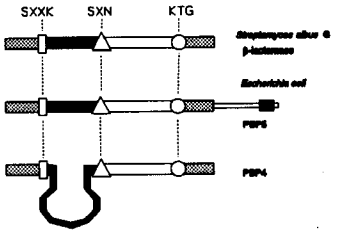


FIG. 4. Positioning of the proposed additional domain in PBP4 in comparison with other members of the family of penicillin-interacting proteins. The distribution and spacing of the three active-site fingerprints SXXK, SXN and KTG in the primary structure is given. The identified membrane anchor in PBP5 is represented as a hatched box.

As can be seen from Fig.3, the overall similarity of PBP4 with ß-lactamases of class A is very low in terms of amino acid identity and similarity but since the fingerprints were clearly picked up by the computer program with a minimum of manipulation, we nevertheless believe that this is significant. In fact this result could only be obtained with the program ARGOS and not with the program of Goad and Kanehisha. This confirms the opinion of the author of ARGOS that this program is very efficient in detecting weak relationships [7]. On the basis of three dimensional structure

comparisons it has been proposed, that ß-lactamases of class A and C and DD-carboxypeptidases of the *Streptomyces* R61 type are products of divergent evolution [22,23], since they show a very low degree of primary structure homology with the exception of some fingerprints being located around the active-site. When however tertiary structures are compared, the homology is very pronounced. It was proposed, that all penicillin-interactive enzymes have a common ancestor [1]. If we assume therefore as a working hypothesis, that the 3D structure of PBP4 resembles the 3D structure of the class A ß-lactamases and search where the additional domain of PBP4 would be located in this model; then we find, that the proposed site of insertion is located on the surface of the ß-lactamase in a very mobile loop. This loop follows immediately the active serine bearing alpha helix (Fig.3 and [26]). It may be possible to test this working hypothesis by making deletions in the additional domain and screening for enzymatically active PBP4 with a decreased molecular weight.

References

[1] **Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J.-M., Kelly, J.A., Boyington, J.C., Moews, P.C. & Knox, J.R.** (1988) The active-site-serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptidase family. Biochem J **250**:313-324.

[2] **Korat, B., Mottl, H., Keck, W.** (1990) Penicillin-binding Protein 4 from *Escherichia coli*: molecular cloning of the *dac*B gene, controlled overexpression and alterations in murein composition. Mol Microbiol **5**:675-684.

[3] **Messing, J.** (1983) New M13 vectors for cloning. Meth Enzymol **101**:21-78.

[4] **Davis, L.G., Dibner,M.D. & Battey, J.F.** (1986) Basic methods in molecular biology. Elsevier, Amsterdam.

[5] **Matsudaira, P.** (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem **262**:10035-10038.

[6] **Lipman, D.J & Pearson, W.R.** (1985) Rapid and sensitive protein similarity searches. Science **227**:1435-1441.

[7] **Argos, P.W.** (1987) A sensitive procedure to compare amino acid sequences. J Mol Biol **193**:385-396.

[8] **Goad, W.B. & Kanehisha, M.I.** (1982) Pattern recognition in nucleic acid sequences. A general method for finding local homologies and symmetries. Nucl Acid Res **10**:247-263.

[9] **Higgins, D.G. & Sharp, P.M.** (1988) CLUSTAL: A package for performing multiple sequence alignments on a microcomputer. Gene **73**:237-244.

[10] **Mulligan, M.E., Hawley, D.K., Entriken, R. & McClure, W.R.** (1984) *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase selectivity. Nucl Acid Res **12**:789-800.

[11] **Stormo, D.G., Schneider, T.D., Gold, L. & Ehrenfeucht, A.** (1982) Computer methods to locate signals in nucleic acid sequences. Nucl Acid Res **12**: 505-519.

[12] **von Heijne, G.** (1986) A new method for predicting signal sequence cleavage sites. Nucl Acid Res **14**:259-268.

[13] **Edelman, A., Bowler, L., Broome-Smith, J-K. & Spratt, B.G.** (1987) Use of a ß-lactamase fusion vector to investigate the organization of penicillin-binding protein 1B in the cytoplasmic membrane of *Escherichia coli*. Mol Microbiol **1**:101-106.

[14] **Jackson, M.E. & Pratt, J.M.** (1987) An 18 amino acid amphiphilic helix forms the membrane-anchoring domain of the *Escherichia coli* penicillin-binding protein 5. Mol Microbiol **1**:23-26.

[15] **Hayashi, S., Hara, H., Suzuki, H. & Hirota, Y.** (1988) Lipid modification of *Escherichia coli* penicillin-binding protein 3. J Bacteriol **170**:5392-5395.

[16] **Klein, P., Kanahisha, M. & DeLisi, C.** (1985) The detection and classification of membrane spanning proteins. Biochim Biophys Acta **869**:197-214.

[17] **Rao, M.J.K. & Argos, P.** (1986) A conformational preference parameter to predict helices in integral membrane proteins.
Biochim Biophys Acta **869**:197-214.

[18] **Eisenberg, D., Schwarz, E., Komaromy, M. & Wall, R.** (1984) Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J Mol Biol **179**:125-142.

[19] **Klein, P., Somorjai, R.L. & Lau, P.C.K.** (1988) Distinctive properties of signal sequences from bacterial lipoproteins. Protein Engineering **2**:15-20.

[20] **Spratt, B.G. & Cromie, K.D.** (1988) Penicillin-binding proteins of gram-negative bacteria.
Rev Infect Disease **10**:699-711.

[21] **Oefner, C., D'Arcy, A., Daly, J.J., Gubernator, K., Charnas,R.L., Heinze, I., Hubschwerlen, C. & Winkler, F.K.** (1990) Refined crystal structure of ß-lactamase from *Citrobacter freundii* indicates a mechanism for ß-lactam hydrolysis.
Nature **343**:284-288.

[22] **Kelly, J.A., Dideberg, O., Charlier, P., Wery, J.P., Libert, F., Moews, P.C., Knox, J.R., Duez, C., Fraimont, Cl., Joris, B., Dusart, J., Frère, J.M., Ghuysen, J.M.** (1986) On the origin of bacterial resistance to penicillin: comparison of a ß-lactamase and a penicillin target. Science **231**:1429-1431.

[23] **Samraoui, B., Sutton, B.J., Todd, R.J., Artymiuk, P.J., Waley. S.G. & Phillips, D.C.** (1986) Tertiary structure similarity between a class A ß-lactamase and a penicillin-sensitive D-alanyl carboxypeptidase-transpeptidase. Nature **320**:378-380.

[24] **Kelly, J.A., Knox, J.R., Haiching, Z., Frère, J.-M. & Ghuysen, J.-M.** (1989) Crystallographic mapping of ß-lactams bound to a D-alanyl-D-alanine peptidase target enzyme. J Mol Biol **209**:281-295.

[25] **Spratt, B.G.** (1977) Properties of the penicillin-binding proteins of *Escherichia coli* K-12.
Eur J Biochem **72**:341-352.

[26] **Herzberg, O. & Moult, J.** (1987) Bacterial resistance to ß-lactam antibiotics: crystal structure of ß-lactamase from *Staphylococcus aureus* PC1 at 0.25 nm resolution. Science **236**:694-701.

[27] **Broome-Smith, J.K., Ioannidis, I., Edelman, A. & Spratt, B.G.** (1988) Nucleotide sequences of the penicillin-binding protein 5 and 6 genes of *Escherichia coli*. Nucl Acid Res **16**:1617.

[28] **Ambler, R.P.** (1980) The structure of ß-lactamases. (1980) Phil Trans R Soc Lond B **289**:321-331.

[29] **Wang, W., Mézes, P.S.F., Yang, Y.Q., Blacher, R.W. & Lampen, J.O.** (1985) Cloning and sequencing of the β-lactamase I gene of *Bacillus cereus* **5/B** and its expression in *Bacillus subtilis*.
J Bacteriol **163**:487-492.

[30] **Madgwick, P.J. & Waley, S.G.** (1987) ß-lactamase I from *Bacillus cereus*.
Biochem J **248**:657-662.

[31] **Hussain, M., Pastor F.I.J. & Lampen, O.** (1987) Cloning and sequencing of the blaZ gene encoding ß-lactamase III, a lipoprotein of Bacillus cereus 569/H. J Bacteriol **169**: 579-586.

[32] **Neugebauer, K., Sprengel, R. & Schaller, H.** (1981) Penicillinase from *Bacillus licheniformis*: nucleotide sequence of the gene and implications for the biosynthesis of a secretory protein in a Gram-positive bacterium. Nucl Acid Res **9**:2577-2588.

[33] **Chan, P.T.** (1986) Nucleotide sequence of the *Staphylococcus aureus* PC1 ß-lactamase gene.
Nucl Acid Res **14**:5940-5940.

[34] **Sutcliffe, J.G.** (1978) Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322.
Proc Natl Acad Sci USA **75**:3737-3741.

[35] **Arakawa, Y., Ohta, M., Kido, N., Fujii, Y., Komatsu, T. & Kato, N.** (1986) Close evolutionary relationship between the chromosomally encoded ß-lactamase gene of *Klebsiella pneumoniae* and the TEM ß-lactamase gene mediated by R-plasmids.
FEBS Lett **207**:69-74.

[36] **Dehottay, P., Dusart, J., De Meester, F., Joris, B., van Beeumen, J., Erpicum, T., Frère, J.-M. & Ghuysen, J.-M.** (1987) Nucleotide sequence of the gene encoding the *Streptomyces albus* G ß-lactamase precursor. Eur J Biochem **166**:345-350.